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Trace element distribution and species fractionation in *Brassica napus* plant

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Trace element status in the parts of the rape plant (roots, shoots, seeds) has been investigated. Roots are able to accumulate large amounts of metals: approximately 2000 mg kg^{-1} Fe; 120 mg kg^{-1} Mn; 30 mg kg^{-1} Zn; 2–8 mg kg^{-1} As, Cr, Ni, Cu and Pb; 0.3–1 mg kg^{-1} Co, Mo and Cd; and 0.1 mg kg^{-1} Ag and Tl (on a dry weight basis). However, the transfer of metals into shoots is limited. The ratios of element contents in shoots and roots are: 10–30% for Ag, Co, Cr, Fe, Mn, Ni and Pb; 50–100% for As, Cd, Cu and Zn; and >110% for Tl and Mo. The transfer of elements into the seeds is even lower except for Cu, Zn, Mo and Tl. The ratios of element contents in seeds and roots are: <20% for Ag, As, Cd, Co, Cr, Fe and Pb; 20–60% for Cu, Mn and Ni; 90% for Tl; and 180–190% for Mo and Zn. The soluble portion of Fe, As and Pb compounds in Tris–HCl buffer (pH = 7.5) represents <10% of total content, whereas that of other elements ranges between 10 and 60%. Extracts of all three parts of plant were submitted to SEC/ICP-MS analysis. While the majority of Co, Cu, Mo, Mn, Ni and Zn compounds is bound in a low-molecular-weight fraction (1–2 kDa), that of As, Ag, Cr, Tl and Pb is present as metal ions or labile complexes. There is one exception: Tl in seed extract is found in three regions of M_r approximately 80, 5 and 2 kDa, respectively. The recovery of chromatographic separation of Tl compounds is approximately 100%. The low-molecular-weight fraction of the seed extract was isolated on preparative-scale SEC and refined by immobilized metal affinity chromatography. The isolated ligands contain large amounts of Cys, Asx, Glx and Gly. Moreover *S*-carboxymethylcysteine was found. No phytochelatins were found by MALDI-MS analysis. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: *Brassica napus*; speciation; trace elements; MALDI-MS; ICP-MS

INTRODUCTION

Rape represents an important crop-plant utilized both for production of oil and as a protein-rich feedstuff as well. Since ancient times rapeseed oil has been used for lighting in oil lamps. Although rape has been grown in Europe since the thirteenth century, its widespread cultivation started during the industrial revolution, when much more oil was needed as a lubricant for steam engines. In the Central Europe rapeseed is still the most important source of vegetable oil. From a botanical viewpoint, rape belongs to the Brassicaceae family. Seeds of various cultivars of three species, *Brassica napus, B. campestris* and *B. juncea*, are used for oil production, depending on the geographical region.[1,2]

It has long been recognized that Brassicaceae plants are able to accumulate appreciable amounts of thallium from the soil,[3] e.g. rapeseed grown on certain French soils (Vault de Lugny, Yonne district) is distinguished by its very high thallium content (18 μg g^{-1} Tl), containing as much as 33 μg g^{-1} Tl (on a dry weight basis).[4] The contents of Tl in plant material and the soil correlate well. Other Brassicaceae plants used as vegetables can also take thallium from the soil, e.g. turnip cabbage (*Brassica oleracea* var. *gongylodes*) and some

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root vegetables (carrot, celery) have shown considerable thallium accumulation from slag-contaminated soil.[5] The accumulation of thallium by turnip cabbage is much more extensive than that of other elements such as As, Cu, Pb and Zn. Thallium transfer from the soil to the plant is affected by its chemical form. More intensive transport of Tl was observed in the case of soil artificially contaminated by thallium (I) sulfate compared with soils with naturally high thallium content.[6] Natural thallium is mainly monovalent and its chemical behaviour is very similar to that of the alkali metals.[7] Therefore the presence of Tl in an ionic Tl^+ form can be assumed. It has also been ascertained that thallium speciation is influenced by the method of its reception by the plant. Gel filtration together with atomic absorption spectrometry has been used for the investigation of Tl and Cd speciation in cytosol of rape leaves.[8,9] Plants cultivated both under physiological conditions and in soil enriched by Tl_2SO_4 were analysed. Almost all Tl in cytosol (70% of total content) was found as uncomplexed ionic Tl^+ species. No Tl^{3+} was detected. In untreated reference plants all thallium was bound to a fraction of relative molecular weight 3.8 kDa. This fraction contained large amounts of Asx, Glx and Gly and no sulfur amino-acids. In both plants, free ionic Cd was found. In cytosol Cd is distributed between two fractions (>30 and 4.4 kDa) independently on thallium.

This study is concerned with the transfer of 13 trace elements from unpolluted soil to roots, shoots and seeds of rape. The method of inductively coupled plasma mass spectrometry (ICP-MS) was chosen for trace element detection since it is very sensitive and allows easy multielemental analysis.[10] Its application for the trace analysis of Tl in materials of plant origin was reported earlier.[11] Besides the determination of total element content in individual parts of rape plants, the present study is focused on mapping the occurrence of soluble forms of trace elements by on-line hyphenation of size exclusion chromatography (SEC) and ICP-MS, and on the characterization of their binding partners as well.

EXPERIMENTAL

Instruments

The ICP-MS measurements were done using Elan 6000 spectrometer (Perkin-Elmer/Sciex, Norwalk, CT, USA) equipped with a Meinhard nebulizer, a cyclonic spray chamber and a Gilson 212 peristaltic pump. Sample decomposition was performed in a UniClever microwave decomposition unit (Plazmatronika-Service, Wroclaw, Poland). pH values of buffer solutions were measured using a pH 03 instrument (Labio, Prague, Czech Republic). The HPLC apparatus used for sample fractionation by on-line SEC/ICP-MS coupling consisted of a Varian Inert 9012 high pressure pump (Varian, Walnut Creek, CA, USA) and two Rheodyne 9010 injectors placed in front of and beyond an SEC column. Both a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden, dimensions 300 × 10 mm, optimum fractionation range 3–70 kDa) and a Fractogel EMD Bio SEC (dimensions 600 × 16 mm, optimal fractionation range 5–1000 kDa, Merck) column were applied. Preparative-scale size exclusion chromatography utilizing the Fractogel column was used for target sample fraction isolation. The apparatus consisted of an LCP 4020 high-pressure pump (Ecom, Prague, Czech Republic), an injector Rheodyne 9010 equipped with a 2 ml PEEK sample loop and a Fractogel EMD Bio SEC column. Samples were freeze-dried using an Alpha 1–2 LD instrument (Martin Christ, Osterode am Harz, Germany). MALDI-MS analyses were performed on a Biflex IV (Bruker Daltonics, Bremen, Germany).

Reagents

Nitric acid used for sample decomposition was of Suprapur® grade (Merck, Darmstadt, Germany). Ag, As, Bi, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Rh, Tl and Zn stock solutions 1000 mg l^{-1} in 2% (v/v) nitric acid (all Merck, Darmstadt, Germany) served for preparation of calibration solutions and an internal standard solution. The mobile phase for chromatographic separation and the extractant were prepared from tris(hydroxymethyl)aminomethane (Tris) (Fluka, Neu-Ulm, Germany) buffered by hydrochloric acid (Suprapur® grade, Merck). Other materials and reagents used during isolation of metal binding peptides were chelating ion exchange resin Chelex 100 (Merck), Sephadex G15 gel (Pharmacia), acetonitrile, 2,5-dihydroxybenzoic acid, trifluoracetic acid (all Fluka) and dithiothreitol (DTT; Merck). De-ionized water (Millipore, Bedford, MA, USA) was used for preparation of all solutions.

Samples and sample extract preparation

Roots and shoots of *Brassica napus* plants were taken in May 2005 in Klínec village (Central Bohemia region), while the corresponding seeds were harvested in August 2005 at the same place. For the elements contents see Table 1. The moisture contents in roots, shoots and seeds were: 69, 82 and 25%, respectively.

Samples were stored at −18 °C, and the roots were carefully washed by distilled water before analyses. Samples of 10 g (roots and shoots) or 5 g (seeds) were crushed in an agate mortar and extracted with 50 ml of 0.02 mol l^{-1} Tris–HCl buffer solution (pH = 7.5) by 1 h shaking in a polypropylene flask. Then the mixtures were centrifuged (20 000*g*, 4 °C, 20 min). The buffer solution was previously purified by passing through the column packed with Chelex 100 resin in NH_4^+ form. Samples of rapeseed flakes and defatted rapeseed meal were provided by Setuza Co. (Ústí nad Labem, Czech Republic).

Analytical methods

Determination of total content of elements

Solid samples (1 g of roots and shoots or 0.5g of seeds) or extracts (10 ml) were decomposed by pressurized microwave digestion in PTFE vessels with 3 ml HNO_3 for 10 min. The

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Table 1. Total content of trace elements in various parts of *Brassica napus* plants and the corresponding soil. Results (mean of six determinations ± expanded uncertainty, $k = 2$) are given in μg g^{-1} of dry matter. The number in the brackets represents the ratios of element contents in proper part of plant and the roots

Element	Roots	Shoots	Seeds	Soil
Ag	0.070 ± 0.006	0.018 ± 0.003 (15%)	0.001 ± 0.0005 (1%)	0.47 ± 0.03
As	2.08 ± 0.14	1.17 ± 0.19 (56%)	0.19 ± 0.11 (9%)	4.8 ± 0.5
Cd	0.40 ± 0.03	0.27 ± 0.04 (69%)	0.05 ± 0.01 (12%)	0.42 ± 0.03
Co	1.27 ± 0.09	0.15 ± 0.02 (12%)	0.04 ± 0.01 (3%)	10.2 ± 0.8
Cr	7.60 ± 0.55	1.80 ± 0.17 (24%)	0.18 ± 0.08(2%)	43.3 ± 3.9
Cu	5.98 ± 0.21	3.06 ± 0.18 (51%)	3.36 ± 0.17 (56%)	18.9 ± 1.1
Fe	2080 ± 120	320 ± 20 (15%)	110 ± 10 (5%)	13700 ± 1800
Mn	116 ± 7	37 ± 2 (32%)	46 ± 2 (40%)	570 ± 55
Mo	0.34 ± 0.03	0.57 ± 0.03 (167%)	0.60 ± 0.05 (176%)	0.44 ± 0.05
Ni	7.43 ± 0.42	2.56 ± 0.28 (34%)	1.79 ± 0.17 (24%)	20.8 ± 1.5
Pb	3.64 ± 0.17	0.44 ± 0.09 (12%)	0.01 ± 0.005 (<1%)	27.9 ± 1.7
Tl	0.067 ± 0.006	0.076 ± 0.008 (114%)	0.063 ± 0.004 (94%)	0.36 ± 0.04
Zn	32.5 ± 1.5	23.8 ± 1.2 (73%)	61.3 ± 2.3 (188%)	69.9 ± 4.6

sample digests were transferred to 50 ml calibrated flask and Rh and Bi solution (internal standards) was added to obtain final concentration of 50 μg l^{-1}. The determination of elements was done by ICP-MS technique with external calibration (details can be found, for example, in Koplík *et al.*[12]).

SEC/ICP-MS analyses

Buffer solution of 0.02 mol l^{-1} Tris–HCl (pH = 7.5) served as the mobile phase; the flow rate was 0.5 or 2 ml min^{-1} in the Superdex 75 and Fractogel columns, respectively. The sample extracts were injected into the SEC column by the first Rheodyne 9025 injector with a 100 or 2000 μl PEEK sample loop, respectively. In the case of analytical scale chromatography the quantification was carried out by post-column injection of calibration solution using the second injector equipped with a 500 μl sample loop. The flow of effluent was conveyed to the nebulizer of ICP-MS. The duration of SEC/ICP-MS analysis was 50 min and the chromatograms consisted of 1000 steps of 3 s each. For details of the procedure see Mestek *et al.*[13]

Isolation of ligands of trace elements

Selected metal-containing fractions of sample extracts were isolated by preparative-scale SEC (for conditions see above). The volume of collected fraction was 6 ml and two independent separation runs were performed in order to combine both portions together. The ligands of trace elements in this sample were refined by adsorption on Chelex-100 resin alternatively in a Cu^{2+} or a Tl^{+} form, which was placed in a 1 ml PE column. Sample flow was 0.5 ml min^{-1}. After washing of the column by water, the adsorbed ligands were eluted by 0.3 mol l^{-1} ammonia solution, and the final volume of eluate was 6 ml. Then 1 ml of antioxidant solution (0.2% DTT) was added to protect sulfhydryl groups of the ligands and after 20 min of incubation at 20 °C the mixture was freeze-dried. Details of the procedure were described in the previous article.[14]

MALDI-MS analyses

The isolated ligands of trace elements were dissolved in 0.1% trifluoracetic acid and desalted by ZipTip with fixed C_{18} reverse phase (Millipore). 2,5-Dihydroxybenzoic acid was used as a matrix for MALDI-MS and the measurement was carried out in positive mode.

Analyses of amino acids

In the case of sample preparation for amino acids determination, the addition of antioxidant was omitted and the sample was desalted by gel filtration on a Sephadex G15 column (dimension 250 × 10 mm) using water as the mobile phase (flow rate 1 ml min^{-1}). After hydrolysis by 6 mol l^{-1} HCl the mixture was analysed by ion exchange chromatography with post-column derivatization by ninhydrin and spectrophotometric detection.

RESULTS AND DISCUSSION

Distribution of trace elements among various parts of the plant

Table 1 shows the distribution of elements in various parts of the rape plant. The amounts of elements in shoots and seeds are related to those in roots. The results are accompanied also by the composition of the soil taken in the place of plant cultivation. It is apparent that the roots are able to take up appreciable amount of trace elements, including toxic ones, from the soil. Simultaneously, roots immobilize toxic elements and restrict their transport to the next part of the plant. As far as the toxic elements are concerned, only As, Cd, and Tl pass

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into green matter. This tissue represents a further barrier: only Tl reaches the seeds. The seeds are also supplied by essential elements (Cu, Mo and Zn). Analysis of industrially produced rapeseed flakes and defatted rapeseed meal originating from another rapeseed sample from different place showed that thallium in seeds is bound to non-lipid constituents, as shown in Table 2.

Trace elements extraction and fractionation

Table 3 contains data concerned with solubility of elements under investigation in 0.02 mol l^{-1} Tris buffer, pH = 7.5. Compounds of Ag, As and Fe originating from all parts of the plant are of low solubility. Also the compounds of Cr and Mn (from roots and seeds) and compound of Pb (from roots and shoots) are slightly soluble. Soluble portions of other elements ranges from 20 to 60% of total element content in particular parts of the plant, except for Mo in the roots (almost all Mo is soluble) and Ni and Pb in the roots (soluble portion 95 and 69%, respectively). The soluble part of Ag, As and Cr in seeds was not evaluated owing to their very low total content.

Table 2. Analyses of rapeseed flakes and defatted meal

	Rape-seed flakes	Defatted rapeseed meal
Moisture content (%)	7.4	7.8
Fat content (%)	42.5	1.9
Tl content (μg g^{-1})	0.161	0.300
Tl content recalculated on fat-less dry matter (μg g^{-1})	0.321	0.332

Individual extracts of plant materials were submitted to on-line SEC/ICP-MS analyses. In addition to distribution of elements into various chromatographic fractions the total amount of element passing the chromatographic column was evaluated as well. The element recovery of chromatographic analysis indicates the percentage of stable complexes of the particular element, i.e. complexes which are not affected by extraction and by chromatographic anylysis. The portion of element eluted in a volume greatly exceeding the total volume of the column corresponds to free metal ions and/or labile complexes. These species are retarded on the column via non-size exclusion effects such as adsorption or ion-exchange; for more details see Koplík *et al*.[12]

As far as the stability of complexes of most elements is concerned, all the analysed samples differed from one another. Ag, As, Cr and Pb in all extracts are present in an ionic form and/or a labile complex form. However, in the case of Tl it is valid only for the extract from shoots. Very minor fractions of stable complexes are typical for Mn (all parts of the plant) and Fe (shoots). The portions of stable complexes of Cu in all extracts, Co in shoots extract and Tl in seeds extract were the highest, while those of other elements represented 20–70% of total amount of element in the particular extract.

The distribution of the elements among individual chromatographic fractions was not the same for all analysed parts of the plant, see Fig. 1. Nevertheless the chromatograms exhibited some patterns. In the case of Ni, Cu, Mo and Zn, the major part of the element in both root extracts (R-E) and shoot extracts (Sh-E) was concentrated in a fraction of low relative molecular weight, 1–2 kDa (t_r = 32 min), while in the seed extract (S-E) these elements passed to a fraction of even lower apparent relative molecular weight, <1 kDa

Table 3. Total contents of elements in extracts (buffer 0.02 mol l^{-1} Tris, pH = 7.5) and the elements' portions passing the SEC column. Results are given in μg g^{-1} (on a dry weight basis)

	Extractable portion[a]			Portion passing SEC column[b]		
Element	Roots	Shoots	Seeds	Roots	Shoots	Seeds
Ag	0.011 (16%)	0.006 (10%)	n.d.	n.d.	n.d.	n.d.
As	0.11 (5%)	0.06 (5%)	n.d.	n.d.	n.d.	n.d.
Cd	0.061 (16%)	0.110 (41%)	0.013 (26%)	0.020 (33%)	0.046 (42%)	0.006 (46%)
Co	0.34 (27%)	0.063 (42%)	0.019(44%)	0.18 (53%)	0.061 (98%)	0.008 (42%)
Cr	0.82 (11%)	0.63 (35%)	n.d.	n.d.	n.d.	n.d.
Cu	2.42 (40%)	1.57 (51%)	1.49 (44%)	2.49 (103%)	1.7 (108%)	1.33 (89%)
Fe	20 (1%)	37 (12%)	10 (9%)	9 (45%)	2 (5%)	4 (40%)
Mn	21 (18%)	10 (28%)	3 (6%)	2 (10%)	0.5 (5%)	0.5 (17%)
Mo	0.36 (104%)	0.33 (57%)	0.24 (40%)	0.07 (19%)	0.22 (67%)	0.12 (50%)
Ni	3.01 (41%)	0.57 (22%)	1.70 (95%)	0.92 (31%)	0.21 (37%)	0.67 (39%)
Pb	0.037 (1%)	0.016 (4%)	0.006 (69%)	n.d.	n.d.	n.d.
Tl	0.014 (21%)	0.047 (61%)	0.013 (20%)	n.d.	n.d.	0.014 (105%)
Zn	12.0 (37%)	7.5 (31%)	8.8 (14%)	5.1 (43%)	2.3 (31%)	2.2 (25%)

[a] Mean of two analyses; the value in parentheses is related to the total content of the element in the original material (see Table 1).
[b] Mean of two analyses; the value in parentheses is related to the extractable portion.
n.d., not detected.

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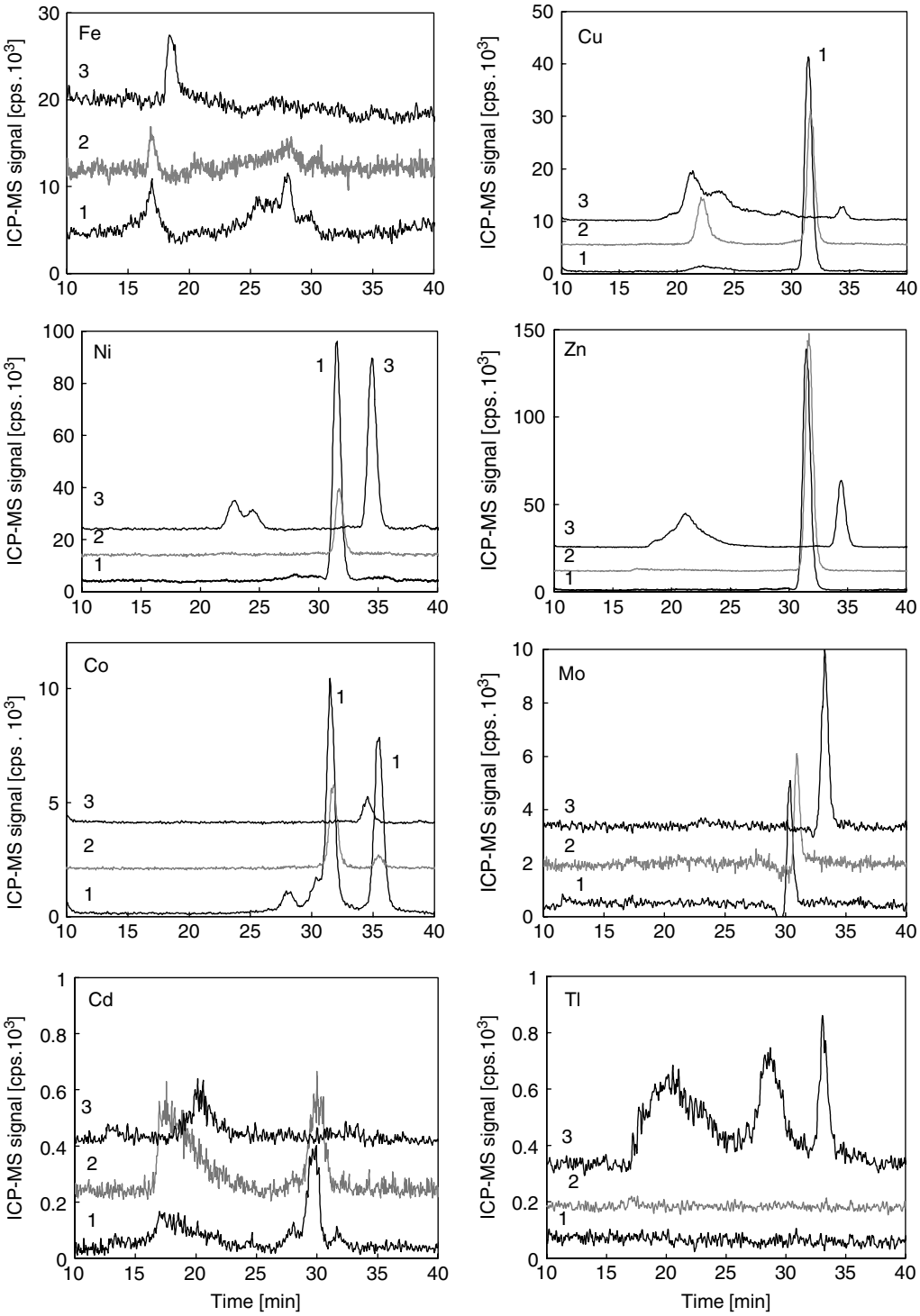

Figure 1. Element specific chromatograms (Superdex 75 HR) of extracts of roots (1), shoots (2, grey line) and seeds (3) of *Brassica napus*.

($t_r = 34–36$ min). Moreover, S-E comprised a wide zone of medium apparent relative molecular weight (20–50 kDa, $t_r = 22–24$ min) of all the above-mentioned elements. On the other hand Cu was the only element bound to a medium-molecular-weight fraction in the Sh-E.

Co in the R-E and the Sh-E was distributed in both low-molecular-weight fractions ($t_r = 32$ min, $t_r = 36$ min), while only the latter Co fraction was found in the S-E. The major part of Cd in R-E was bound to a compound of apparent relative molecular weight 10 kDa ($t_r = 29$ min), but only a

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minor amount of high apparent relative molecular weight (>150 kDa, $t_r = 16–18$ min) Cd compounds was present. The portion of these compounds tended to increase in the Sh-E; moreover in the case of S-E the medium molecular weight Cd compounds disappeared and only a high-molecular-weight fraction (100 kDa, $t_r = 20$ min) was detected.

The total amount of Tl in the R-E and the Sh-E occured as an ionic and/or a labile complex form. Stable complexes were found only in the S-E where Tl was quite uniformly distributed among three fractions: 1 kDa ($t_r = 34$ min), 10 kDa ($t_r = 29$ min) and 100 kDa ($t_r = 19$ min).

Fe was the only element that did not form low-molecular-weight compounds. Medium molecular weight compounds (5–20 kDa, $t_r = 25–30$ min) were involved, especially in the R-E; their portion in the Sh-E was lower and they were not present in the S-E at all. Compared with this, high-molecular-weight iron compounds ($t_r = 17$ min, >150 kDa) were involved in all studied extracts.

Sample extract S-E was subjected to further investigation by on-line preparative-scale SEC/ICP-MS hyphenation. During these experiments sulfur and phosphorus were also monitored as constituents of potential ligands of trace elements: phytates and cysteine-rich peptides. The used Fractogel column was found to be able to mutually separate a subfraction of phosphorus and sulfur species and that of trace elements species involved in the low-molecular-weight fraction; however, this separation was poor, see Fig. 2.

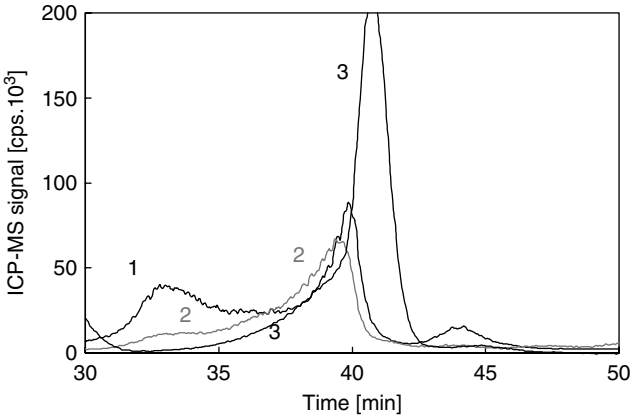

Figure 2. Detail of SEC/ICP-MS chromatograms (Fractogel EMD Bio SEC) of low-molecular-weight species of P (1), S (2, grey line) and metals (3) in rapeseed extract.

Characterization of ligands of trace elements

The low-molecular-weight fraction of seed extract, which contained substantial amounts of trace metals, was isolated using preparative-scale SEC and, after purification on Chelex 100 resin with immobilized metal ions, was submitted to further analyses. This type of chromatography can isolate only compounds with a strong affinity to metals. Two modifications of the process were applied. The first one used Chelex 100 in a Cu^{2+} form since Cu complexes were found to be the most stable. The second process was based on Chelex 100 in a Tl^{+} form in order to find contingent differences between complexation of Tl and other metals. The ligands of trace elements isolated from such sample were analysed for amino acid composition and by MALDI-MS. For the relative contents of amino acids see Table 4. The contents of not reported amino acids were below 4%. Both samples exhibited similar amino-acid patterns characterized by high content of Cys, Gly and acidic amino acids (Asp, Glu) and/or their amides (Asn, Gln). Moreover they contain another dicarboxylic amino acid, *S*-carboxymethylcysteine. The MALDI-MS spectra of Cu^{2+}-purified and Tl^{+}-purified ligands of trace elements were almost identical; see Fig. 3. They contained two peaks: the first at 2260 Da and the second at 2699 Da.

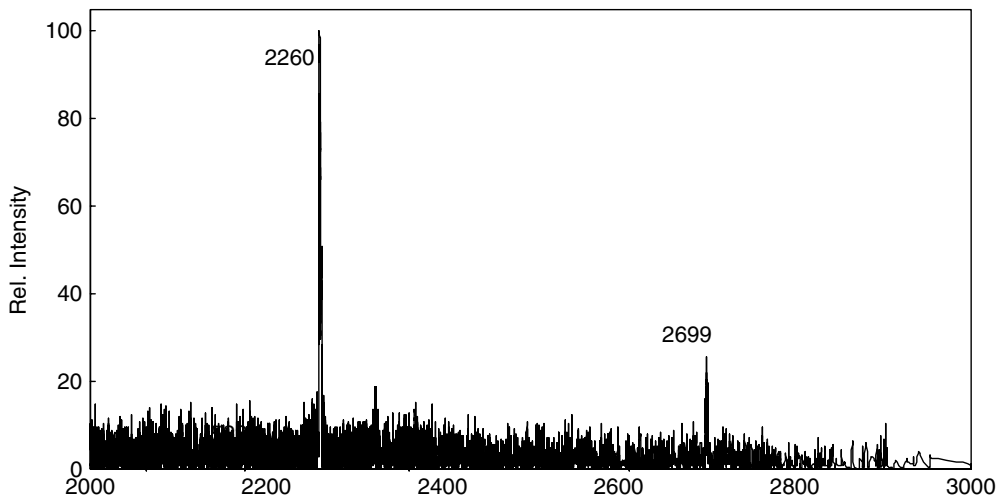

Figure 3. MALDI MS spectra ($M - H^{+}$) of low-molecular-weight fraction of rapeseed extract refined on Chelex-100 in a Cu^{2+} cycle.

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Table 4. Relative contents of amino acids (mol%) in the ligands of trace elements isolated from low-molecular-weight fraction of rape seeds extract

Amino acid	Refined on Chelex 100 in a Cu^{2+} cycle	Refined on Chelex 100 in a Tl^{+} cycle
Cys	10	23
CM Cys[a]	12	9
Asp + Asn	18	5
Glu + Gln	17	25
Gly	12	22
Val	5	<4
Lys	5	<4

[a] *S*-carboxymethylcysteine.

CONCLUSION

High contents of Gly, Asx and Glx found in ligands of trace elements isolated from low-molecular-weight fraction of rape seed extract were in good agreement with previous data.[8,9] In addition, some sulfur-containing amino acids were found as well. Nevertheless, the chromatogram of this low-molecular-weight fraction in Fig. 2 convincingly shows that sulfur as well as phosphorus does not accompany metals. Therefore it can be assumed that the sulfur-containing amino acids found originate from insufficiently separated S and P subfractions which precede the analysed metal subfraction, and that sulfur compounds play a less important role in the chelating of metals. This hypothesis is also supported by the fact, that any peak found in MALDI-MS-spectra does not correspond to phytochelatins, which are often considered as the main metal-binding compounds in plant materials.[15] The absence of phytochelatins in seeds of the plants is not surprising as these metal detoxifying peptides are induced by increased toxic metals levels and their lifetime in plant cells is quite short. Aspartic and glutamic acids and/or their amides are probably the constituents responsible for metal binding in plants grown under physiological conditions. Similar amino acid composition of ligands of trace elements was also ascertained in the case of buckwheat and amaranth flours.[14] Aspartic and glutamic are known as components of many metaloproteins[16] and they can bind metals by ionic coupling and coordination link using O atoms as well. For more details about the bonding of metals on bio molecules see, for example, Metzler.[17] Searching for the structure of isolated ligands will be the subject of our next study.

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